

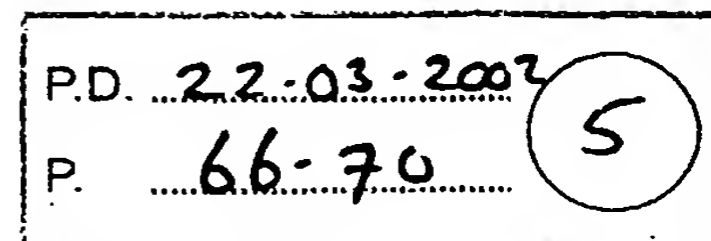
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Active Site Mutations of Recombinant Deacetoxycephalosporin C Synthase

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Site-directed mutagenesis of active site residues of deacetoxycephalosporin C synthase active site residues was carried out to investigate their role in catalysis. The following mutations were made and their effects on the conversion of 2-oxoglutarate and the oxidation of penicillin N or G were assessed: M180F, G299N, G300N, Y302S, Y302F/G300A, Y302E, Y302H, and N304A. The Y302S, Y302E, and Y302H mutations reduced 2-oxoglutarate conversions and abolished (<2%) penicillin G oxidation. The Y302F/G300A mutation caused partial uncoupling of penicillin G oxidation from 2-oxoglutarate conversion, but did not uncouple penicillin N oxidation from 2-oxoglutarate conversion. Met-180 is involved in binding 2-oxoglutarate, and the M180F mutation caused uncoupling of 2-oxoglutarate from penicillin oxidation. The N304A mutation apparently enhanced *in vitro* conversion of penicillin N but had little effect on the oxidation of penicillin G, under standard assay conditions. © 2002 Elsevier Science (USA)

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Deacetoxycephalosporin C synthase (DAOCS) catalyzes the ring-expansion of penicillin N to deacetoxycephalosporin C, during the biosynthesis of cephem antibiotics in *Streptomyces clavuligerus* and other prokaryotes (1–4). The enzyme is part of the family of iron(II), 2-oxoglutarate-dependent oxygen-

Abbreviations used: DAC, deacetylcephalosporin C; DACS, deacetylcephalosporin C synthase; DAOC, deacetoxycephalosporin C; DAOCS, deacetoxycephalosporin C synthase; DAOC/DACS, deacetoxy/deacetylcephalosporin C synthase; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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ases (5) that includes other enzymes involved in cephalosporin biosynthesis, such as deacetylcephalosporin C synthase (DACS) and deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS) (6). The former enzyme catalyzes the hydroxylation step subsequent to ring expansion in *S. clavuligerus*, (7) whilst the latter catalyzes both the expansion and hydroxylation reactions in *Cephalosporium acremonium* (8–10).

Understanding of the catalytic mechanism of DAOCS has been recently advanced by a combination of X-ray crystallographic (4, 11) and site-directed mutagenesis studies. The latter studies have demonstrated that Arg-258 is important in controlling the selection of 2-oxoglutarate as the 2-oxoacid co-substrate (12), and that several other arginine residues (especially Arg-160, -162, and -266) are involved in penicillin substrate binding (13). In addition, residues at the C-terminus, although not necessarily bound to the prime substrate at the point of oxidation, appear to be important for maintaining coupling between 2-oxoglutarate conversion and penicillin oxidation (14). Here we report site-directed mutagenesis on other residues present in the active site of DAOCS (4).

MATERIALS AND METHODS

Materials. Chemicals were obtained from the Sigma-Aldrich Chemical Co. or E. Merck and were of analytical grade or higher. Reagents were also supplied by Amersham Biosciences (protein chromatography systems and columns); Bohringer-Mannheim (ATP); MBI (1Kb and 100 bp DNA gel markers); Bio-Rad (mutagenesis reagents); New England Bio-Labs (enzymes for molecular biology); Novagen (vectors); Sigma-Genosys (mutagenesis primers); Phenomenex (HPLC columns); Promega (Wizard Plus miniprep DNA purification system, Wizard Plus SV miniprep DNA purification system); Stratagene (competent cells, PCR Script vector, QuikChange mutagenesis kit); and Qiagen Ltd. (RNase A).

Site-directed mutagenesis. The M180F mutant was constructed using the unique site elimination (USE) system (Amersham Biosciences) (15). The remaining point mutants (G299N, G300N, Y302F/G300A, Y302S, Y302H, Y302E, N304A) and the 304-NVI-305 insertion mutant were constructed using the method of Kunkel (16, 17) (Table 1). Automated DNA sequencing (Department of Biochemistry,

TABLE 1
Primers Used to Construct the DAOCS Mutants

Mutation	Primer sequence	Method
M180F	5'-GAGCAGCCCCTGCGGTCCGCGCCGCACTACGAC-3'	USE
G299N	5'-GTAGTTGCCGTTGATCCAATCC-3'	Kunkel
G300N	5'-CACGTAGTTGTTCCCGATCCA-3'	Kunkel
Y302F/G300A	5'-ATGTTACGAAGTTGCCCC-3'	Kunkel
Y302S	5'-ATGTTACGGAGTTGCCCC-3'	Kunkel
Y302E	5'-GATGTTACCTCGTTGCCCCC-3'	Kunkel
Y302H	5'-TGTTACAGTGGTTGCCCCC-3'	Kunkel
N304A	5'-GCGCGGATGGCACGTAGTT-3'	Kunkel
304-NVI-305	5'-TGTGCGGCGGATGACGTTACGTAGTT-3'	Kunkel

Note. Bold residues denote the mutation site. USE, unique site elimination. Selection primers: (*pst* I to *nco* I) 5'-CGTGACACCA-CGATGC*CATGGGCAATGGCAACAACG-3'. Kunkel: Method developed by Kunkel (16, 17).

University of Oxford) confirmed the sequences of all mutants before sub-cloning into the pET11a or pET24a vectors. The required plasmids were transformed into *E. coli* XL1 Blue and *E. coli* BL21(DE3) and grown as previously reported (4).

Protein purification and assays. Mutant enzymes were purified to >85% purity (by SDS-PAGE analysis) as previously described (13), and analyzed by circular dichroism spectroscopy (4). 2-Oxoglutarate conversion assays were conducted as previously described (18), using 0.1 mM penicillin N or 10 mM penicillin G as substrates. An equivalent volume of water was added when no penicillin substrate was used. Deacetoxycephem products were assayed using the reported HPLC assays (4). Approximately 0.15 mg of enzyme was used in each assay. Steady-state kinetic analyses were carried out by the reported procedures (4). ¹H NMR (500 MHz) spectroscopy was used to confirm the presence of the expected cephem products (4). Note that the cofactor/(co)substrate conditions for the different types of assay are not identical. Moreover, the results of 'discontinuous' assays can be difficult to interpret (19, 20).

RESULTS AND DISCUSSION

Construction, Purification, and Assaying of Mutants

The M180F mutation was made in order to probe the functional significance of this residue, which is known to be involved in 2-oxoglutarate binding but is also close to the iron and proposed prime substrate binding site (4, 11). The G299E, G300E, Y302S, Y302F/G300A, Y302E, Y302H, and N304A mutations were made in order to probe their effects on penicillin substrate binding. The planned Y302F mutant also contained an additional mutation, G300A. An insertion mutant (304-NVI-305), with a valine inserted between Asn-304 and Ile-305, was also made to probe the relationship between these two residues. Automated DNA sequencing confirmed the presence of the required mutations.

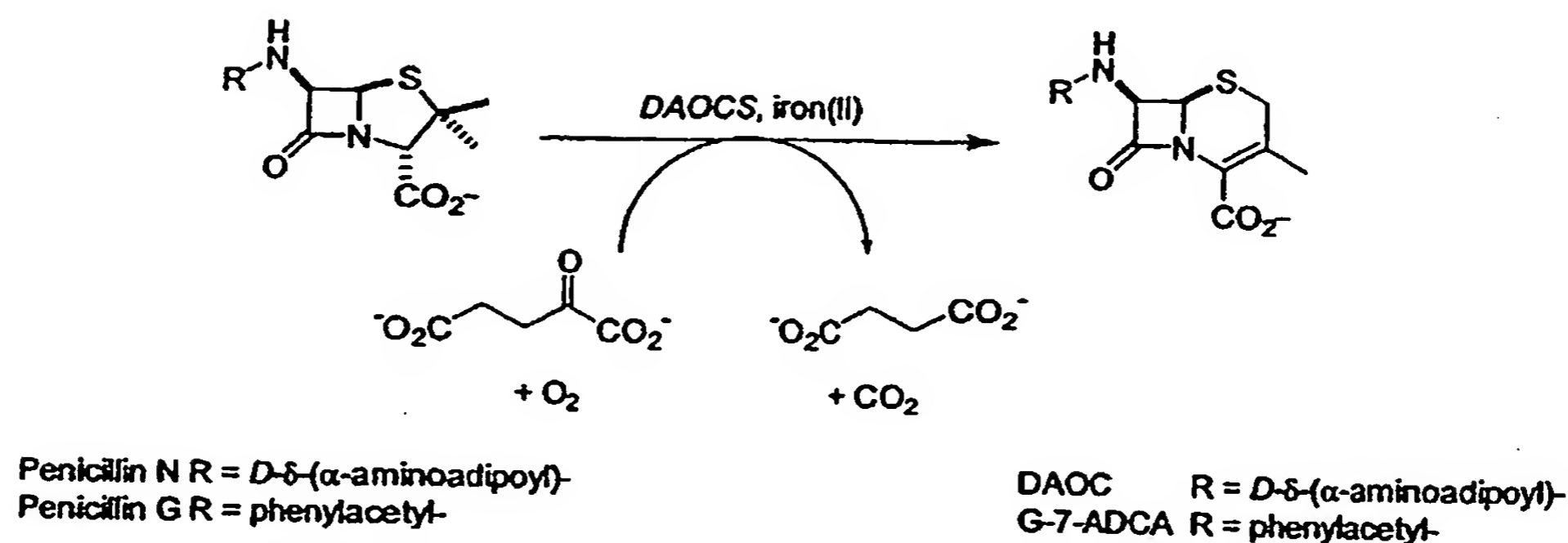
All mutants were purified by anion-exchange and hydrophobic interaction chromatographies to >85% purity (as judged by SDS-PAGE analysis). Circular dichroism analysis of all mutants suggested that no gross changes in conformation in the mutants compared to the wild-type enzyme had occurred. The activity of all mutants was initially analyzed using 'discontinuous' assays for both 2-oxoglutarate and

penicillin conversion (Table 2). Two penicillin substrates were assessed: penicillin N [the natural substrate, possessing the D-(α -amino adipoyl-) side-chain], and penicillin G (an unnatural substrate, possessing the phenylacetyl side-chain) (Scheme 1). Modification of DAOCS to efficiently accept the latter substrate offers the possibility of directly fermenting deacetoxycephems with hydrophobic side-chains making their isolation more efficient than at present. Subsequently, steady-state kinetic analysis was carried out (Table 3) for selected mutants catalyzing significant penicillin oxidation. Note that these kinetic assays measure penicillin turnover and that the stoichiometry of 2-oxoglutarate conversions may exceed that of the penicillin substrate if the two oxidative steps are uncoupled. Enzyme inactivation during catalysis may

TABLE 2
Activity Assays for Wild-Type and Mutant DAOCS
with 0.1 mM Penicillin N or 10 mM Penicillin G

	Enzyme	Penicillin N		Penicillin G	
		2-OG	HPLC	2-OG	HPLC
1	Wild-type	100	100	79	65
2	M180F	67	44	60	<2
3	G299N	N/D	N/D	35	9
4	G300N	N/D	N/D	64	59
5	Y302S	<5	<2	<5	<2
6	Y302F/G300A	52	33	46	6
7	Y302E	N/D	N/D	30	<2
8	Y302H	N/D	N/D	14	<2
9	N304A	148	177	41	91
10	304-NVI-305	N/D	N/D	26	3

Note. Assays measured 2-oxoglutarate conversion (18) and penicillin conversion by HPLC (4). 2-Oxoglutarate conversions were corrected for that in the absence of a penicillin substrate; i.e., they represent stimulation of 2-oxoglutarate utilization. Results are normalized to penicillin N conversion by the wild-type enzyme (specific activity: 26 nmol/min/mg enzyme), and are based on at least duplicate readings. Standard deviations are ca. 15 and 10% for 2-oxoglutarate and penicillin conversion, respectively. N/D, not determined.



SCHEME 1. The ring expansion reaction catalyzed by DAOCS. G-7-ADCA, phenylacetyl-7-aminodeacetoxycephalosporanic acid.

also occur, and this may be accelerated under uncoupled reaction conditions (21).

Activity of C-Terminal Mutants

Glycines-299 and -300 have been previously proposed to be part of a hinge section allowing movement of the C-terminal region (4), which was proposed to form a 'lid' over the DAOCS active site during catalysis (14). The planned Y302F mutant also contained an additional mutation, G300A. Since the conformation of the probably mobile C-terminus may be different in solution from that in the crystal structure (14), it is difficult to assess the effect of this inadvertent mutation.

The G299N and G300N mutations were therefore made to investigate the effects of substitution of a bulkier (and hydrophilic) side-chain into this conserved Gly-Gly hinge region (4). The G299N mutation dramatically reduced penicillin G conversion, suggesting that the presence of a glycine residue at this location is important for the function of the C-terminus (4, 14).

TABLE 3

Kinetic Parameters for Penicillin Conversion by Wild-Type DAOCS and Mutants by HPLC Assay

Mutant	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ s ⁻¹)
(a) Using penicillin N as substrate			
Wild-type	0.036 ± 0.007	0.05 ± 0.007	1388
Y302F/G300A	0.032 ± 0.005	0.008 ± 0.0014	250
N304A	0.032 ± 0.002	0.08 ± 0.004	2500
(b) Using penicillin G as substrate			
Wild-type	0.7 ± 0.1	0.05 ± 0.006	71
G300N	0.86 ± 0.24	0.029 ± 0.002	34
Y302F/G300A	0.22 ± 0.02	0.0008 ± 0.0001	3.6
N304A	1.4 ± 0.2	0.09 ± 0.008	64

Note. Values are reported ± standard deviation.

Penicillin G oxidation is reduced to a much greater extent than 2-oxoglutarate conversion (Table 2, entry 3), suggesting that the major effect of this mutation is to uncouple the two reactions. In contrast, the G300N mutation only reduced activity to a small extent, and coupling between 2-oxoglutarate and penicillin G oxidation is maintained (Table 2, entry 4). The Y302F/G300A double mutation has a relatively small effect on activity (Table 2, entry 6), suggesting that the effect of the G300A mutation is relatively small.

The insertion of a valine residue between Asn-304 and Ile-305 abolished penicillin G conversion (Table 2, entry 10). The inserted residue presumably occupies the same relative position as Ile-305 in the wild-type enzyme, but also results in displacement in the position of subsequent C-terminal residues. Previous mutagenesis studies have shown the importance of the C-terminus (14), and the side-chains of some residues within it (13), in maintaining coupling between penicillin oxidation and 2-oxoglutarate conversion. The present study implies that some C-terminal residues are more important than others, most strikingly illustrated by the relative effects of mutation of Gly-299 and Gly-300.

Mutations Located in the Penicillin Binding Site

Two residues (Tyr-302 and Asn-304) within the C-terminus and close to the predicted penicillin substrate binding site were also targeted for study. The Y302S mutation abolished oxidation of both penicillin N and G (Table 2, entry 5). 2-Oxoglutarate conversions were not enhanced compared to the levels observed in the absence of a penicillin substrate, suggesting that (productive) penicillin binding was abolished by this mutation. In contrast, conversion of 2-oxoglutarate by the Y302F/G300A mutant was stimulated in the presence of penicillins N and G (Table 2, entry 6). Some uncoupling between penicillin N oxidation and 2-oxoglutarate conversion was observed, and this was much more pronounced when penicillin G was used as

substrate. In the case of the Y302E and Y302H mutants (Table 2, entries 7 and 8) this uncoupling was essentially complete. The similar behaviour of the Y302F/G300A, Y302E and Y302H mutants (together with the results for the G300N mutant) suggests that the presence of the G300A mutation has a minor effect on DAOCS activity.

Steady-state kinetic analysis of the Y302F/G300A mutant revealed an interesting effect on the conversion of penicillin N and G. In both cases k_{cat} was significantly decreased whilst there was relatively little (if any) change in K_m . These results suggest Tyr-302 is more important for the oxidation of the penicillin rather than for its initial binding. However, in the absence of a crystal structure of DAOCS with a bound penicillin substrate it is difficult to assign a specific role for this residue.

The N304A mutation appears to slightly increase the rate of both 2-oxoglutarate and penicillin N conversion (22) under the standard *in vitro* assay conditions (Table 2, entry 9). Steady-state kinetic analysis (Table 3) showed that the N304A mutation apparently results in an increase in k_{cat} for the conversion of penicillin N with K_m unchanged. In the case of penicillin G the apparent K_m has doubled, but k_{cat} has also been increased. Thus, the overall catalytic efficiency for penicillin G is similar to the wild-type enzyme.

The side-chain of the N304 residue has been proposed to bind to the amide bond linking the penicillin nucleus with the side-chain (4), but these results suggest that this is unlikely. Recently, Chin *et al.* reported the effects of the N304L mutation (23), which also apparently increased the rate of conversion of penicillin G, ampicillin and amoxycillin under a variety of cofactor conditions. Asn-304 is located relatively close to the C-terminus of the enzyme, modifications of which are known to enhance levels of penicillin conversion (14). Our results, together with those of Chin *et al.* (23), suggest that Asn-304 mutations exert their effects by modulating C-terminal function. The results also support the suggestion that the C-terminus of DAOCS has evolved to maximise coupling between penicillin oxidation and 2-oxoglutarate conversion rather than or as well as maximising catalytic activity (13).

Methionine-180 Has a Role in both 2-Oxoglutarate and Penicillin Oxidation

Methionine-180 is located in the 2-oxoglutarate co-substrate binding site of DAOCS. Previous studies have indicated that substituting Met-180 for a selenomethionine residue had little effect on catalytic efficiency (4). Isopenicillin N synthase is an oxidase closely related to DAOCS in which the analogous residue to Met-180 is Phe-211 (*A. nidulans* enzyme) (24). The M180F mutation of DAOCS (Table 2, entry 2)

resulted in clear uncoupling of 2-oxoglutarate conversion from penicillin oxidation from penicillin G oxidation. In the case of penicillin N (the natural substrate) this uncoupling is much less pronounced, if occurring at all. These results imply that the side-chain of Met-180 is also involved in productive binding of the penicillin substrate.

The uncoupling of 2-oxoglutarate conversion from penicillin (G) oxidation may cause a non-productive conformation of the co-substrate side-chain at some point during catalysis, as was proposed to explain the uncoupling effect of the R258Q mutation (12). Alternatively, the M180F mutation may directly or indirectly influence the conformation of the penicillin substrate. Analysis of the available DAOCS crystal structures show that the side-chain of Met-180 is adjacent to the carboxylate and keto group of 2-oxoglutarate, and projects towards Arg-162, which is involved in binding the penicillin carboxylate (13). Modelling studies suggest that mutation of Met-180 to a phenylalanine residue will result in steric interaction with the side-chain of Arg-162, thus impairing penicillin binding. In the case of penicillin G, the high level of uncoupling may be a reflection of the higher K_m value for this substrate in the wild-type enzyme (4, 19).

CONCLUSIONS

The results in this study are in general agreement with the catalytic mechanism and binding model for penicillin substrate previously proposed (4, 14). The results demonstrate that the effect of individual mutations can differ for different penicillin substrates. Consideration of this factor is an important in efforts to re-engineer DAOCS (and related oxygenases) to accept alternative prime substrates. It seems likely that contributions from several specific amino acid residues and the general conformation of the enzyme, cofactors and substrates will control the substrate selectivity of DAOCS. The development of fermentation protocols involving the ring expansion of penicillin G to cephem products is desirable, as these may be used as feed-stock materials for the production of semi-synthetic antibiotics.

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